IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re-application of:

Tony Marcel *et al.*

Group art Unit: 1647

Serial N°: 10/024,535

Examiner: Wegert, Sandra L.

Filed: December 21, 2001

For: Therapeutic methods and compositions for the treatment of impaired interpersonal and behavioural disorders.

DECLARATION UNDER RULE 132

Hon. Commissioner of Patents and Trademarks

WASHINGTON D.C. 20231

Sir :

I, Catherine ROUGEOT, residing at 39 Route de Choisel, 78460 Chevreuse (France) ;

Declare and say :

I am citizen of France.

I am PhD in Neurobiology and Pharmacology, graduated from University Pierre et Marie Curie Paris VI.

I am currently head of the Laboratory of Pharmacology of Neuro-Endocrine Regulations of Institut Pasteur (France).

I am an inventor of the present patent application and I am aware that the Examiner alleged that evidences would be missing showing that SMR1 peptides would have activity in humans or that the human homologs of SMR1 exist.

I previously demonstrated that the SMR1 QHNPR pentapeptide binds *in vivo* to NEP (Neutral endopeptidase or Neprilysin) in rats and that QHNPR inhibits substance P breakdown by NEP *in vitro,* in rat renal membranes (QHNPR is now called "sialorphin"; and see Rougeot C. et al., 2003. Sialorphin, a natural inhibitor of rat membrane-bound endopeptidase that displays analgesic activity. P.N.A.S., vol 100, 8549-8554).

I herein provide *in vitro* results demonstrating that the SMR1 pentapeptide, QHNPR, has inhibitory activity on membrane preparations of human cells expressing NEP, the receptor for the QHNPR pentapeptide.

The *LNCaP cell line* has been described as expressing NEP as well as other members of the metalloectopeptidase family. LNCaP (adenocarcinoma, ATCC (CRL-1740)) is a prostate epithelial cell line.

By using Northern blot and immunocytochemical analyses, it has been confirmed under my supervision that the LNCaP cells were able to express NEP (mRNA and cell surface protein) in defined medium culture conditions (*i.e.*, RPMI containing insulin, transferin and selenium, GIBCO) and after 48 hours induction by DHT (10^{-9} M dihydrotestosterone).

Furthermore, in an experimental model of static incubations of membrane preparations originating from these cells, the parameters allowing to analyze endoproteolysis of substance P mediated by human NEP in the conditions of initial velocity measurement have been defined (*i.e.* 98 ± 10 pM/min/µg LNCaP cell membrane proteins, n=12).

The LNCaP membrane activity of substance P endoproteolysis was inhibited in the presence of specific synthetic NEP inhibitors, such as

thiorphan (58 ± 7%, n=13 for maximum inhibitory potency at 1 µM) or in the presence of specific synthetic DPPIV (dipeptidyl peptidase IV) inhibitor, such as DPPIV inhibitor II (42 ± 8%, n=3 for maximum inhibitory potency at 10 µM). In contrast, bestatin (25 µM) and captopril (10 µM) which block the aminopeptidase (AP-N, APB) and angiotensin-converting enzyme (ACE) activities, respectively, did not significantly inhibit substance P hydrolysis by cell surface ectopeptidases. Thus, this indicated that in the experimental conditions used, the extra-cellular breakdown of substance P was mainly caused by the NEP and DPPIV endopeptidase activities located at the surface of these cells.

Accordingly, inhibition of human NEP-mediated endoproteolysis of substance P by the SMR1 peptide QHNPR was assayed under my supervision as follows:

Cell membrane preparations: human LNCaP cell pellet was collected and harvested in 10 volumes (vol./wt.) of ice-cold 50 mM Tris-HCl buffered at pH 6.5. A first centrifugation at 1,200xg and 5°C for 5 min allowed removing the cellular debris and the nuclei in the pellet while the resulting supernatant was submitted to light sonication (20 sec at 4°C). A second centrifugation at 100,000xg and 5°C for 30 min concentrated the cell membranes in the pellet, which was superficially washed with cold Tris-HCl buffer, resuspended in fresh buffer, aliquoted and stored at –80°C while waiting to be used as enzyme source. The NEP proteins in cell membrane suspensions were determined by using the Bio-Rad DC protein assay.

Physiological substrates: Modified tritiated substance P [(3,43H)Pro2-Sar9-Met(O2)11]-Substance P (Perkinelmer-NEN) was used as NEP-sensitive substrate.

Measurement of NEP activity using substance P substrate: Hydrolysis of the substance P substrate was measured by monitoring its metabolism rate by NEP under conditions of initial velocity measurement and in the presence and absence of the SMR1 pentapeptide QHNPR with concentrations ranging from 1 to 50 µM (n=25). The QHNPR peptide was added to the preincubation medium.

Using microsorb tubes, the standard reaction mixture consisted of cell membranes in 50 mM Tris-HCl pH 6.5 containing 0,1% BSA (200 µl final volume). The substance P substrate (60 nM final concentration containing 100 nCi 3H-Substance P) was added after preincubation for 10 min and the hydrolysis was carried out for 20 min at 25°C in a constantly shaken water bath. The reaction was terminated by cooling to 4°C and adding HCl (0.3 N final concentration). The reaction tubes were then centrifuged (4,500xg for 15 min at 4°C) and the products of the reaction were isolated and quantified according to their differential hydrophobic characteristics. By using C-18 Sep-Pak cartridges (Waters), the 3H metabolites were isolated by elution (4 ml) with H_2O-.1% TFA and then with 25% methanol-0.1% TFA and the intact tritiated substrate was eluted with 75-100% methanol-0.1% TFA.

Statistical analysis: Analysis of Variance (ANOVA) for linear regression analysis of Substance P hydrolysis inhibition *vs.* concentration of pentapeptide QHNPR was performed and the IC50 of QHNPR on human NEP was calculated.

The results of these experiments show that QHNPR concentration-dependently inhibited substance P catabolism by cell surface human NEP (LNCap cell line). The percentage of inhibition of Substance P hydrolysis as a function of QHNPR concentration is displayed on Figure 1.

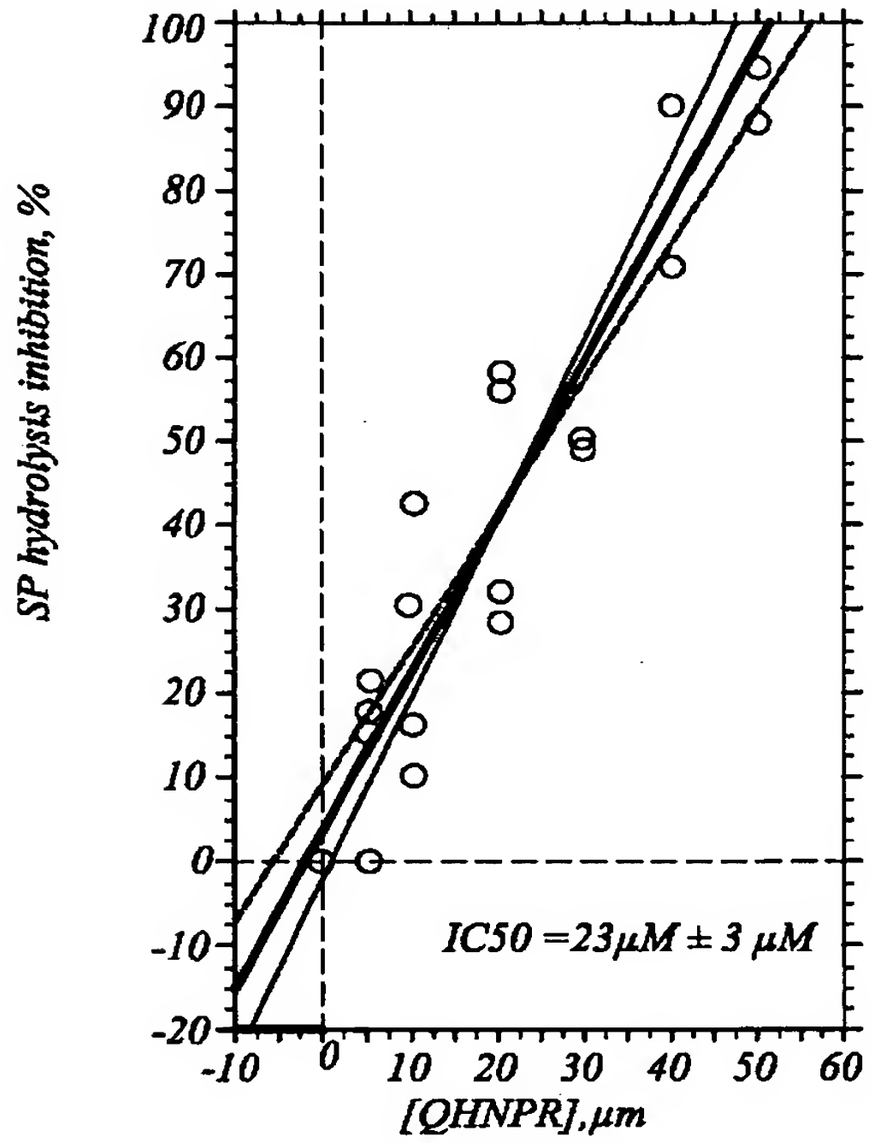

Figure 1: effect of QHNPR pentapeptide on the breakdown of substance P by human NEP

An ANOVA for linear regression analysis of the percentage of inhibition of Substance P hydrolysis as a function of QHNPR concentration was performed and it was found :

Substance P hydrolysis inhibition (%) = 3.69 + 1.853 x [QHNPR] (μM);

$R^2 = 0.92$, n = 25, with $p \leq 0.0001$.

Table 1: ANOVA Substance P hydrolysis inhibition, % *vs.* [QHNPR], μM

Source	Degrees of Freedom	Sum of squares	Mean Square	F value	p value
Model	1	26635.267	26635.267	263.746	<0.0001
Error	23	2322.733	100.988		
Total	24	28958.000			

Table 2: Regression summary Substance P hydrolysis inhibition, % *vs.* [QHNPR], µM

Number	25
Missing	0
R	0.959
R^2	0.920
Adjusted R^2	0.916
Residual standard deviation	10.049

The inhibitory concentration 50% of QHNPR pentapeptide on substance P catabolism by cell surface human NEP (LNCap cell line) was thus determined as 23 ± 3 µM.

Therefore, these results demonstrate that the SMR1 pentapeptide, QHNPR, has inhibitory activity on membrane preparations of human cells expressing NEP, which is the receptor for the QHNPR pentapeptide. These data thus provide evidence that the SMR1 peptides have activity in humans.

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The undersigned Declarant declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true ; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this day of 28 March 2006